APOPTOTIC ENDONUCLEASE EndoG INDUCES ALTERNATIVE SPLICING OF CASPASE-2

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Caspase-2 (Casp-2) is an enzyme that regulates the development of apoptosis upon alternative splicing of its mRNA. The long form of Casp-2 (Casp-2L) promotes apoptosis while the short form (Casp-2S) has decreased enzymatic activity and inhibits the development of apoptotic processes. However, very little is known about the mechanism of Casp-2 alternative splicing. Several endonucleases are known to participate in this process. The aim of this study was to determine the role of EndoG in regulation of Casp-2 alternative splicing. Strong correlation between expression levels of EndoG and Casp-2 splice-variants was found in $CD4^+$ and $CD8^+$ human T lymphocytes. Such correlation increased after incubation of these cells with etoposide. Increased expression of Casp-2S was determined during EndoG over-expression in $CD4^+$ T-cells, after EndoG treatment of cell cytoplasm and nuclei and after nuclei incubation with EndoG digested cell RNA. Casp-2 alternative splicing was induced by a 60-mer RNA oligonucleotide in naked nuclei and in cells after transfection. The identified long non-coding RNA of 1016 nucleotides is the precursor of the 60-mer RNA oligonucleotide. Based on the results the following mechanism has been proposed. Casp-2 pre-mRNA is transcribed from the coding DNA strand while long non-coding RNA is transcribed from the template strand of the Casp-2 gene. EndoG digests long non-coding RNA and produces the 60-mer RNA oligonucleotide complementary to the Casp-2 pre-mRNA exon 9 and intron 9 junction place. Interaction of the 60-mer RNA oligonucleotide and Casp-2 pre-mRNA causes alternative splicing.

Key words: EndoG; alternative splicing; caspase-2; T lymphocytes

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INTRODUCTION

Caspase-2 (Casp-2) belongs to the family of cysteine proteases, which cleave the peptide bond formed by aspartic acid in the amino acid sequence of protein substrates. Caspases are initiators, mediators, and effector molecules in the development of apoptosis and play a crucial role in the regulation of cell death [1]. Casp-2 is one of the most evolutionarily conserved among other initiator and effector caspases [2].

The process of alternative splicing (AS) of the Casp-2 pre-mRNA has a significant effect on the activity of the resulting forms of the enzyme. The full-length Casp-2L (the long form of 435 residues) consists of the subunits p19 and p12, which are involved in the catalytic activity of the enzyme [3]. The Casp-2S splice variant (the short form) contains a non-coding exon at the 5' end of the mRNA. Its inclusion in mature mRNA results in translation initiation from coding codon 2 and loss of the N-terminal 31-mer peptide [4]. As a result of AS, Casp-2S loses the p12 coding sequence, exon 9, and enzymatic activity. Casp-2L induces cell death, whereas Casp-2S increases survival [5].

Two factors are known to be involved in the regulation of the AS pre-mRNA Casp-2. The protein hnRNPA1 (heterogeneous nuclear ribonucleoprotein A1) promotes the formation of Casp-2S, whereas the splicing factors ASF (alternative splicing factor) and SF2 (splicing factor 2) induce Casp-2L [6]. DNA damage by various agents, oxidative stress, and heat shock of cells cause changes in the proportion of splice variants in cells [7–11].

Previously, Casp-2 was shown to be associated with several apoptotic endonucleases [12, 13], destroying cellular DNA in the final stages of apoptosis [14]. Endonuclease G (EndoG) is a site-specific endonuclease that selectively cleaves double-stranded DNA at poly(dG) sequences. A distinctive feature of EndoG is that this enzyme has RNase activity [15]. We have previously shown that EndoG is involved in the synthesis of splicing-switching oligonucleotides, the so-called EndoG-produced oligonucleotides (EGPO), which are able to modulate telomerase and nuclease activity [16–18]. The aim of this work was to investigate the relationship between EndoG and Casp-2 in human T lymphocytes.

MATERIALS AND METHODS

Blood Collection, Selection and Cultivation of T Lymphocytes

Venous blood samples from healthy donors were collected in tubes containing the anticoagulant K_3EDTA (Greiner Bio-One, Austria). Mononuclear cells were

Abbreviations used: Casp-2 – caspase-2; Casp-2L – the long form of caspase-2; EndoG – endonuclease G; Casp-2S – the short form of caspase-2; EGPO – EndoG produced oligonucleotide; AS – alternative splicing; ncRNA – non-coding RNA.

isolated from the blood by gradient centrifugation using Ficoll Lympholite-H (Cedarlane, Canada). Isolation of the $CD4^+$ cell fraction was performed by magnetic selection using a CD4+ isolation kit, human (Miltenyi Biotec, Germany) according to the manufacturer's protocol. Isolation of the $CD8^+$ T-cell fraction was performed using a CD8+ Isolation Kit, human (Miltenyi Biotec). Cell culture was performed according to the previously described protocol [19]. The resulting cells were seeded at a concentration of 5×10^5 cells per 1 ml of medium. We used culture medium RPMI-1640 (Life technologies, USA) with 10% FBS (Fetal Serum Bovine, Gibco, USA), growth stimulants 100 U/ml IL-2 (R&D Systems, USA), 5 μg/ml anti-CD3 antibody (MedBioSpectrum, Russia), 2 μg/ml anti-CD28 antibody (eBiosciences, USA), and antibiotics 50 U/ml penicillin and 50 mg/ml streptomycin (Sigma, USA). Culturing was performed in a CO_2 incubator at 37°C, 5% CO_2, and 90% humidity.

Incubation with Etoposide and Transfection of Cells

To induce Casp-2S expression, $CD4^+$ and $CD8^+$ T lymphocytes were cultured in the presence of a non-lethal concentration (5 μM) of the topoisomerase II inhibitor etoposide (Sigma) for 24 h.

Transfection of $CD4^+$ T cells was performed with one of the pEndoG-GFP plasmids or the control pGFP plasmid (custom synthesized by Clontech, USA) based on the pGFP-N1 vector [19]. Transfection was performed using Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's protocol. Transfection efficiency (typically 90–99%) was assessed by flow cytometry by counting the number of GFP-positive cells. To transfect cells with a 60-mer EGPO nucleotide (EndoG-produced oligonucleotide, nucleotide produced by EndoG) or control RNA (Table 1, Synthol, Russia), Oligofectamine Reagent (Invitrogen) was used according to the manufacturer's protocol. EGPO and control RNA contained bases with phosphorothioate linkages to protect against the action of nucleases.

Isolation of Cellular Organelles and Their Processing with RecEndoG

Cytoplasm was obtained by the method described by Laukova et al [20]. Nuclei were isolated according to the method described by Pravdenkova et al [21]. We have previously described the procedure in detail [22]. Cytoplasm and nuclei were incubated with recombinant human EndoG ENDOG (Human) Recombinant Protein (Abnova, USA) at 37°C.

Quantitative PCR and Determination of Enzyme Activity

Total RNA was isolated from cells, nuclei, and cytoplasm using a total RNA isolation kit (Biolabmix, Russia) according to the manufacturer's protocol. Reverse transcription was performed using reagents from the MMLV RT kit, and real-time PCR was performed using qPCRmix-HS SYBR (both reagents from Evrogen, Russia) according to the manufacturer's protocols. The primers used (Synthol) are listed in Table 2. The detailed protocol has been previously described by us [23]. *18S* (a cytoplasmic housekeeping gene) and *LMNB1* encoding lamin B1 (a nuclear housekeeping gene) were used as the reference genes. Expression data were presented as normalized mRNA levels of the genes studied, based on the average expression value of two reference genes [19].

Table 1. Oligonucleotides used for cell transfection and incubation with nuclei

Name	Sequence 5′–3′
EGPO	GGCACCUCCUUCUGUUCACUGCUGCCACCGCCUCUCUUGCUCUGUAAGUGUCUCCCAAUG
Control RNA	AUGUGCCGUAGGUGAGGCCUCACGUUCGUUAAACGGAUUCCGUCAGGAUAGGAGAUACUC
EGPO	G*G*C*A*C*CUCCUUCUGUUCACUGCUGCCACCGCCUCUCUUGCUCUGUAAGUGUCUCC*C*A*A*U*G
Control RNA	A*U*G*U*G*CCGUAGGUGAGGCCUCACGUUCGUUAAACGGAUUCCGUCAGGAUAGGAGA*U*A*C*U*C

Note: * - phosphorothioate bond.

Table 2. Primers used for quantitative PCR

Target	Forward primer (5′–3′)	Reverse primer (5′–3′)	Amplification size, b.p.	Annealing temperature °C
EndoG	AATTGAGCTCCGCACCTACGTGAT	AGGATGTTTGGCACAAAGAGCAGC	167	55
Casp-2 general	GCATGTACTCCCACCGTTGA	TGCTCAACACCAGTGCTAGG	242	60
Casp-2L	AGCTCTTTGACAACGCCAA	CAGGAACCTCGTTTGGTGTT	262	58
Casp-2S	CCGTGGAGGTGCTATTGG	TCGGCAACTTTTCTTTACCG	165	57
ncRNA	CGTGCACCACCATATCCTGT	GGGCACCAGAGTTAATGAAGC	101	60
EGPO	ACCTCCTTCTGTTCACTGCTG	CCCATGCATTGGGAGACACTT	63	59
18S	GGATCCATTGGAGGGCAAGT	ACGAGCTTTTTAACTGCAGCAA	91	64
LMNB1	GATTGCCCAGTTGGAAGCCT	TGGTCTCGTTAATCTCCTCTTCATACA	152	60

The enzymatic activity of Casp-2 was determined using the Caspase 2 Assay Kit Fluorometric (Abcam, USA) according to the manufacturer's protocol. The procedure has been described in detail by us previously [23].

DNA and RNA Cleavage by recEndoG Enzymes or RNases

DNA was isolated using the LIRA reagent and the D-cells kit for isolation of DNA from animal and bacterial cells (Biolabmix). RNA was isolated as previously described. The resulting DNA and RNA were incubated with 0.3 μg recEndoG in 50 mM Tris-HCl, pH 7.9, 1 mM $CaCl_2$, 10 mM $MgCl_2$, 5 mM β-mercaptoethanol for 20 min, 40 min and 60 min at 37°C. RNA was incubated with 500 units of RNase 1 (Invitrogen) and RNase A (Invitrogen) for 60 min according to the manufacturer's protocol. Samples were then incubated with Proteinase K (Biolabmix) to inactivate recEndoG or RNases. Each of the 24-mer DNA oligonucleotides (Table 3) was added to the enzyme-treated RNA and incubated for 30 min at 37°C to form non-covalent DNA-RNA complexes that block EGPO activity [19, 24].

Identification of ncRNAs

Total RNA was isolated from cells, nuclei, and cytoplasm using the RNeasy Mini Kit (Invitrogen). Libraries for sequencing were prepared using the TruSeq Small RNA Library Preparation Kit (Illumina, USA) according to the manufacturer's protocol. Sequencing was performed on a MiSeq System sequencer (Illumina) and data analysis was performed as described by Lopez et al [25].

Table 3. DNA oligonucleotides used to block EGPO activity

#	Sequence 5′–3′
1	TCGAGAGTGAAAGGACAAACGAGG
2	GGACAAACGAGGAGAGTCCACGAT
3	AGAGTCCACGATAACCTAGGGAAC
4	AACCTAGGGAACCCGTGGAGGAAG
5	CCGTGGAGGAAGACAAGTGACGAC
6	ACAAGTGACGACGGTGGCGGAGAG
7	GGTGGCGGAGAGAACGAGACATTC
8	AACGAGACATTCACAGAGGGTTAC
9	ACAGAGGGTTACGTACCCCACACG
10	GTACCCCACACGACCCTGAACCCG
11	ACCCTGAACCCGTCGGGTACCGAG
12	TCGGGTACCGAGAGTCCGACCAGT

Statistical analysis

Statistical analysis of the results was performed using the Student's *t*-test with the program Statistica 9.0. Results are presented as mean ± standard deviation. Differences were considered statistically significant at $p \leq 0.05$. To investigate the relationship between EndoG expression levels and Casp-2 splice variants, $CD4^+$ and $CD8^+$ T cells were ranked according to the level of EndoG expression and divided into two groups. Cells with an EndoG expression level below the median were considered as the cells with low EndoG expression, while cells with an EndoG expression level above the median were considered as the cells with high EndoG expression. In these groups, Pearson correlation analysis of the expression levels of EndoG and *Casp-2* splice variants was performed using Statistica 9.0.

RESULTS

EndoG Expression Correlates with the Expression of Casp-2 Splice Variants

To determine the relationship between EndoG and Casp-2 splice variants, the levels of EndoG mRNA and Casp-2 splice variants were assessed in $CD4^+$ and $CD8^+$ T lymphocytes from twelve donors by using quantitative PCR. Incubation of cells with 5 μM etoposide (a non-lethal concentration selected by us in preliminary experiments) resulted in a significant increase in EndoG expression in most samples of $CD4^+$ and $CD8^+$ T cells (Fig. 1). Etoposide increased the expression of both total Casp-2 and each of the splice variants. Cells were ranked according to the level of EndoG expression. The median was 0.296 for $CD4^+$ T lymphocytes and 0.218 for $CD8^+$ T lymphocytes. After incubation with etoposide, the median was 0.480 and 0.503 for $CD4^+$ and $CD8^+$ T lymphocytes, respectively.

The correlation coefficients of EndoG expression with Casp-2 splice variants are shown in Table 4. The results obtained are consistent with data showing that etoposide causes an increase in Casp-2S expression [3, 26, 27]. As the correlation between EndoG expression and Casp-2 splice variants was strongest in $CD4^+$ T cells, these cells were selected for further work.

Table 4. Correlation coefficients of EndoG mRNA and Casp-2 splice variants

Splice variant of Casp-2	Low EndoG mRNA levels		High EndoG mRNA levels	
	$CD4^+$	$CD8^+$	$CD4^+$	$CD8^+$
Casp-2L (Control)	0.701 (n=6)	0.554 (n=7)	0.722 (n=6)	0.540 (n=5)
Casp-2L (Etoposide)	0.747 (n=6)	0.697 (n=6)	0.836 (n=6)	0.709 (n=6)
Casp-2S (Control)	0.612 (n=6)	0.646 (n=6)	0.608 (n=6)	0.677 (n=6)
Casp-2S (Etoposide)	0.815 (n=6)	0.735 (n=6)	0.702 (n=6)	0.706 (n=6)

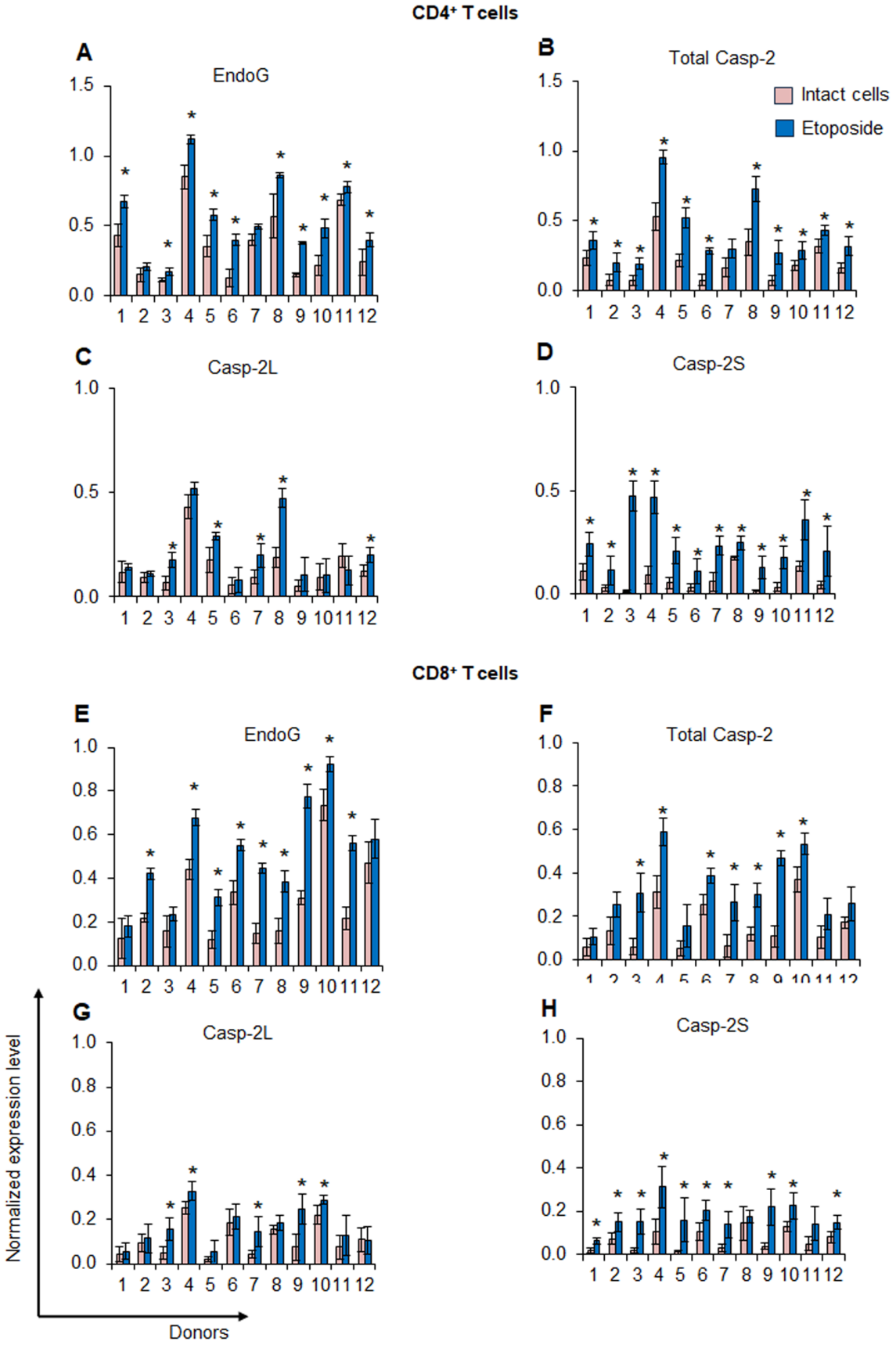

Figure 1. Relationship between EndoG, Casp-2S, and Casp-2L mRNA levels in T lymphocytes before and after etoposide treatment. Levels of (**A**, **E**) EndoG and (**B**, **F**) total Casp-2, (**C**, **G**) Casp-2L, and (**D**, **H**) Casp-2S mRNA in $CD4^+$ and $CD8^+$ T lymphocytes. Gene expression levels are normalized to the expression of reference 18S rRNA. * $p \leq 0.05$ relative to intact cells.

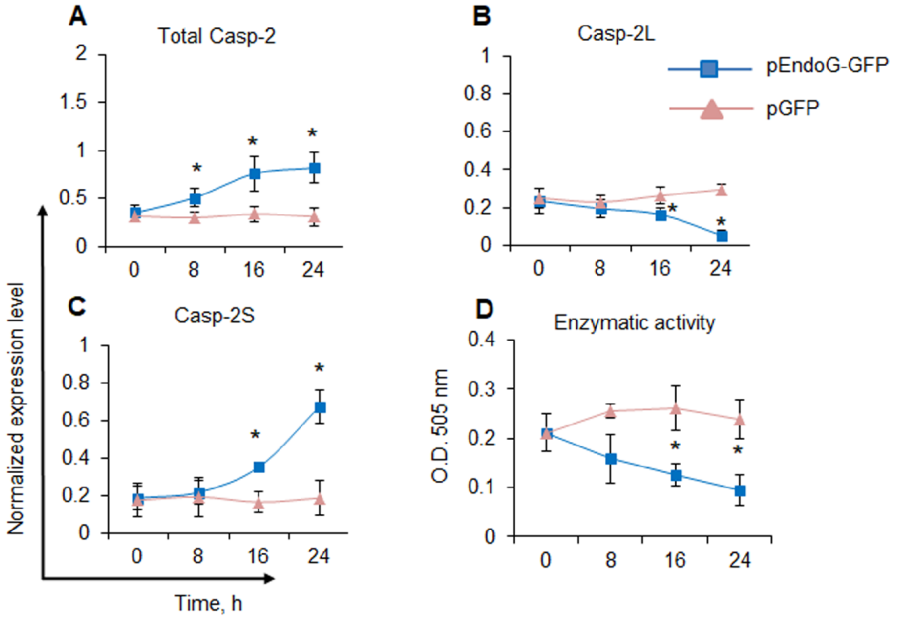

Figure 2. mRNA levels of Casp-2 splice variants in human CD4⁺ T lymphocytes transfected with pEndoG-GFP or pGFP. Levels of (**A**) total Casp-2, (**B**) Casp-2L, and (**C**) Casp-2S mRNA in CD4⁺ cells 24 h after transfection, measured by quantitative PCR. (**D**) Casp-2 enzymatic activity in transfected cells. n=4, * $p \leq 0.05$ compared to cells transfected with pGFP.

Increased Casp-2 AS with EndoG Overexpression

To determine the possible role of EndoG in the induction of AS by Casp-2, CD4⁺ T cells were transfected with the pEndoG-GFP plasmid. The transfection efficiency was high, approaching 98–99% within 8 h of transfection. Transfection of cells with the EndoG gene was accompanied by a significant increase in the amount of total Casp-2 mRNA (Fig. 2A). Analysis of the mRNA levels of the Casp-2 splice variants showed that, despite an increase in total Casp-2, there was a decrease in the amount of *Casp-2L* mRNA (Fig. 2B) with a concomitant increase in Casp-2S mRNA (Fig. 2C). In cells transfected with pEndoG-GFP, the enzymatic activity of Casp-2 decreased significantly already 16 h after transfection (Fig. 2D). No changes in the levels of Casp-2 splice variants and enzymatic activity were observed when cells were transfected with the control plasmid pGFP. The result of this experiment suggests that the decrease in the enzymatic activity is most likely due to a decrease in the amount of Casp-2L, since it is this form that has catalytic activity.

Induction of AS Casp-2 in the Cytoplasm and Nuclei as a Result of the recEndoG Action

The apoptotic endonuclease EndoG is normally localised in the intermembrane space of mitochondria and is internalized into the nucleus during the development of apoptotic processes of DNA hydrolysis [28]. Nuclei and cytoplasm were isolated from CD4⁺ T cells. Subsequent qPCR analysis showed that Casp-2S mRNA was completely absent in the nuclei (Fig. 3). Similar amounts of Casp-2L were found in the cytoplasm and nuclei. Incubation of nuclei and cytoplasm with recEndoG for one hour resulted in appearance of Casp-2S in the nuclei and a significant increase in the cytoplasm. At the same time, there was a decrease in the expression of Casp-2L. Since Casp-2S was absent in the nuclei of CD4⁺ T cells and only appeared in the presence of recEndoG, the nuclei of these cells became a model for further investigation of the effect of EndoG on Casp-2 AS.

EndoG has previously been shown to synthesize small RNAs that modulate AS (EGPO) as a result of its RNase activity [16–18]. To demonstrate that EndoG acts similarly on Casp-2, total DNA and RNA were isolated from CD4⁺ T cell nuclei and incubated with recEndoG or control non-specific RNases (RNase A or RNase 1). Nuclease-treated DNA and RNA were purified from proteins and incubated with CD4⁺ T cell nuclei. The amount of Casp-2 was analyzed by quantitative PCR. This experiment has shown that it is RNA, and not DNA, subjected to recEndoG cleavage that is able to induce Casp-2S in the nuclei of these cells (Fig. 3C). Neither RNase A nor RNase 1 induced this effect. Changes in the expression of Casp-2 splice variants during incubation of nuclei with recEndoG-treated RNA were time-dependent (Fig. 3D). Increasing the time of RNA treatment caused a significant increase in the amount of Casp-2S mRNA and a decrease in the amount of Casp-2L mRNA.

Identification and Expression of the EGPO RNA Oligonucleotide Produced by EndoG

A search of GenBank for a sequence similar to the Casp-2 exon 9 yielded no results. Therefore, we have hypothesized that the EndoG-induced

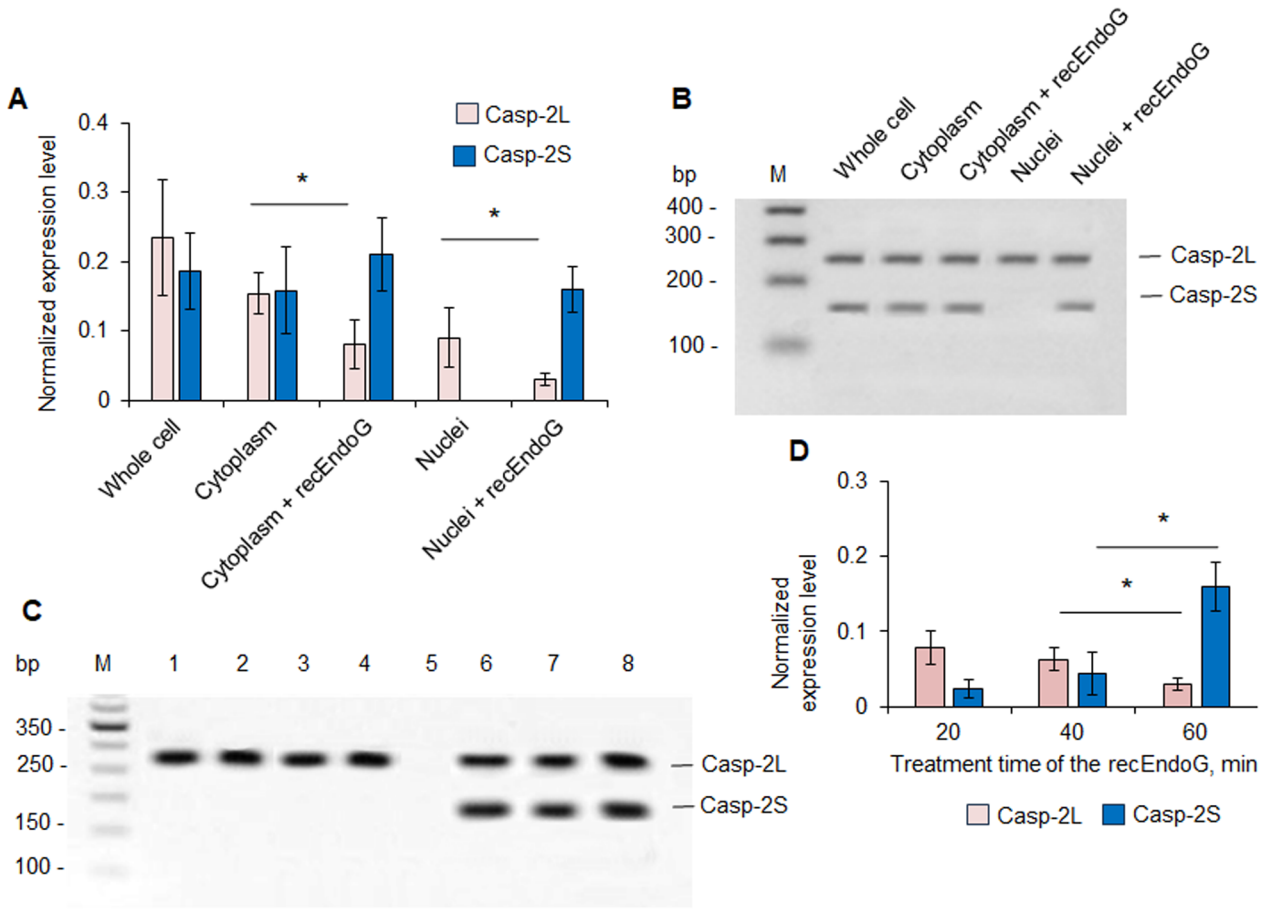

Figure 3. Casp-2 splice variant mRNA levels in the cytoplasm and nuclei of CD4+ T cells after recEndoG treatment. (**A**) Casp-2 splice variant mRNA levels. (**B**) Agarose gel electrophoresis of PCR amplifications of Casp-2 splice variants. (**C**) Induction of *Casp-2* AS in $CD4^+$ T cell nuclei by PCR amplification of Casp-2 splice variants: *1* – nuclei; *2* – nuclei after incubation with recEndoG-treated DNA; *3* – nuclei after incubation with RNase A-treated RNA; *4* – nuclei after incubation with RNase 1-treated RNA; *5* – recEndoG-treated RNA; *6* – nuclei after incubation with RNA treated with recEndoG for 20 min; *7* – nuclei after incubation with RNA treated with recEndoG for 40 min; *8* – nuclei after incubation with RNA treated with recEndoG for 60 min. (**D**) Levels of mRNA of Casp-2 splice variants in nuclei incubated with RNA treated with recEndoG. n=4, * $p \leq 0.05$. M – molecular weight marker.

oligonucleotide (EGPO) capable of inducing Casp-2 AS is derived from a long non-coding RNA (ncRNA) synthesized from a DNA strand complementary to the Casp-2 transcription strand. To test this hypothesis and determine the length of EGPO, recEndoG-digested RNA from $CD4^+$ T cell nuclei was incubated with each of the 24-mer DNA oligonucleotides complementary to the intron 8, exon 9, and intron 9 regions of the potential Casp-2 ncRNA and added to $CD4^+$ T cell nuclei (Fig. 4A). These oligonucleotides are able to form non-covalent complexes with EGPO and block its action [24]. PCR analysis showed that splicing was blocked by four oligonucleotides, allowing the size of EGPO to be determined at the level of 60 nucleotides (Fig. 4B). The nucleotide sequence of EGPO is shown in Figure 4A.

A quantitative PCR study of EGPO expression showed that transfection with EndoG caused a significant increase in the amount of EGPO (Fig. 4C). An increase in EGPO expression was also found in the cytoplasm treated with recEndoG (Fig. 4D). EGPO was not detected in intact nuclei and appeared after incubation with recEndoG. These data are consistent with the absence of the *Casp-2S* splice variant in the nuclei of intact $CD4^+$ T cells and its appearance after transfection with the EndoG gene or treatment of nuclei with recEndoG.

Induction of AS Casp-2 by Synthesized EGPO

To confirm that EGPO is able to activate the AS pre-mRNA Casp-2, $CD4^+$ T cell nuclei were incubated with artificially synthesized EGPO or a control non-specific RNA of the same size. This experiment showed that synthesized EGPO was able to activate AS in a dose-dependent manner at a concentration of 10^{-7} M and higher (Fig. 5A–D). With increasing EGPO concentration, an increase in the level of Casp-2S mRNA and a decrease in the level of Casp-2L mRNA were observed. Thus, EGPO probably acts as an endogenous splice-switch oligonucleotide and induces Casp-2 AS.

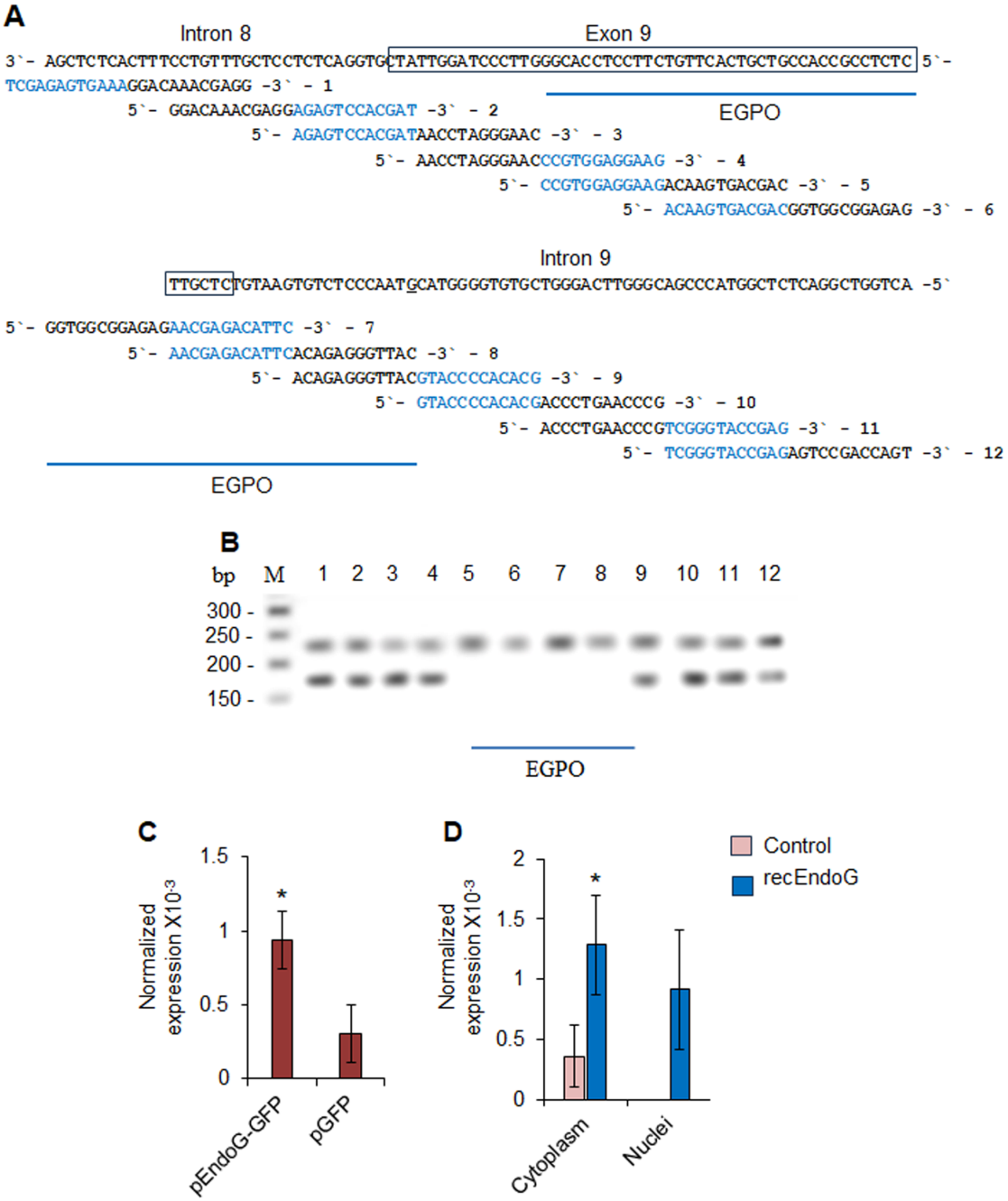

Figure 4. Regulation of EGPO AS and EGPO expression. (**A**) Schematic position of antisense DNA oligonucleotides relative to *Casp-2* ncRNA and EGPO. (**B**) Agarose gel electrophoresis of *Casp-2* PCR amplification products in nuclei incubated with DNA oligonucleotides. M is a molecular weight marker. EGPO expression levels (**C**) in CD4$^+$ T cells transfected with pEndoG-GFP or pGFP plasmids and (**D**) in the cytoplasm and nuclei of cells after recEndoG treatment. Exon 9 is boxed.

Since EGPO induces AS *Casp-2* in cell-free experiments (i.e. nuclei and cytoplasm), to test that EGPO is active in cells, CD4$^+$ T cells were transfected with synthesized EGPO or control RNA. Quantitative PCR showed that the level of Casp-2S mRNA significantly increased in cells transfected with EGPO (Fig. 5E). At the same time, a significant decrease in the amount of Casp-2L mRNA was observed. The change in the proportion of Casp-2 splice variants was accompanied by a decrease in enzymatic activity in the samples (Fig. 5F).

Identification and Expression of ncRNAs

To identify the ncRNA from which EndoG excises EGPO, total RNA sequencing analysis was performed. An ncRNA was identified that overlaps the end of intron 6, the entirety of exons 7, 8 and 9, introns 7 and 8, and the beginning of intron 9 of the Casp-2 pre-mRNA strand (Fig. 6A).

Quantitative PCR was used to determine the level of ncRNA in cells transfected with EndoG-GFP or GFP plasmids and in the nuclei and

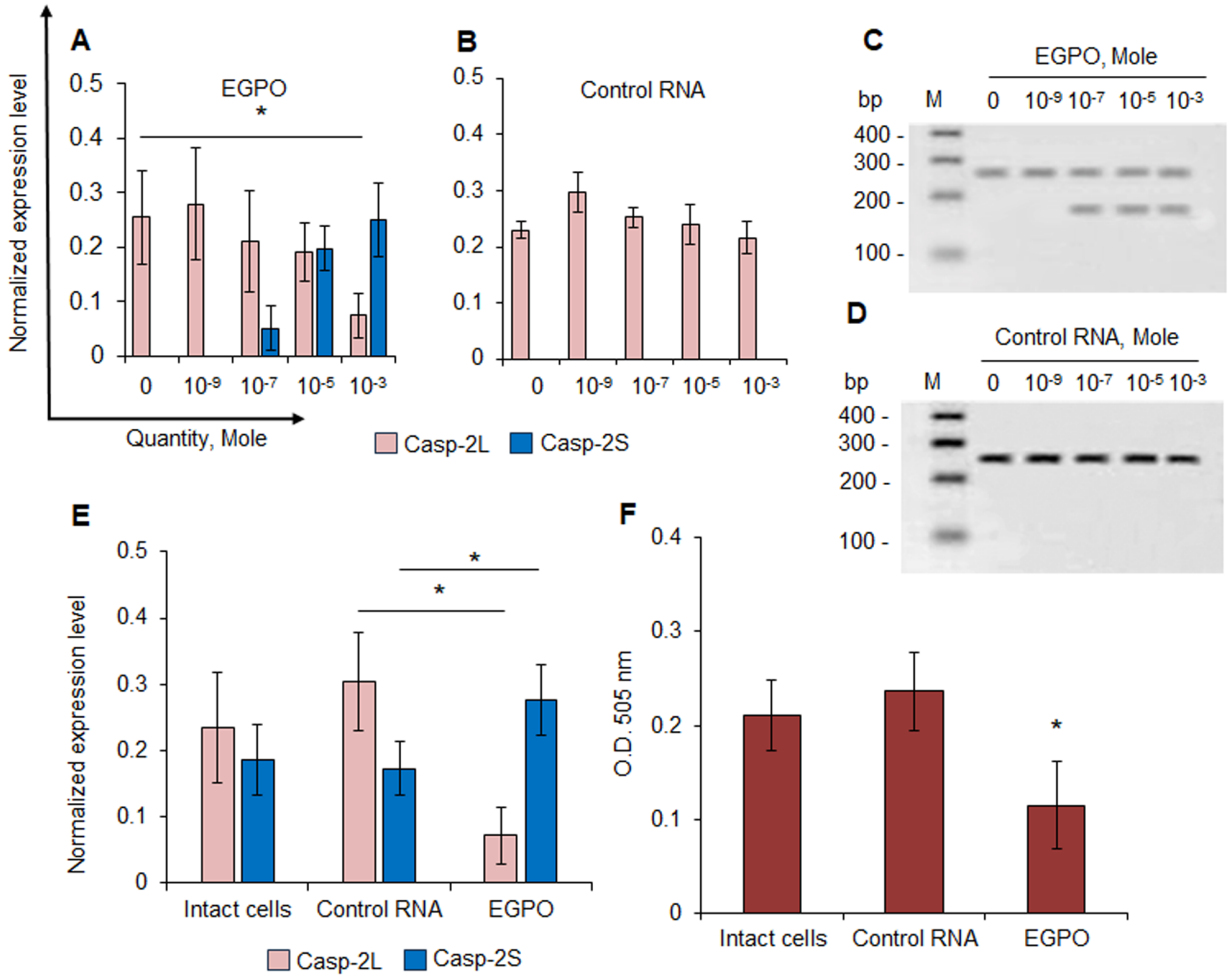

Figure 5. Casp-2 splice variant mRNA levels after EGPO treatment. Expression levels of Casp-2 splice variants in nuclei after treatment with (**A**) EGPO or (**B**) unspecific control RNA. Agarose gel electrophoresis of PCR amplification of Casp-2 splice variants in nuclei after treatment with (**C**) EGPO or (**D**) control nonspecific RNA. (**E**) Casp-2 splice variant mRNA levels in cells 24 h after transfection. (**F**) Casp-2 enzymatic activity in transfected cells. n=4, * – $p \leq 0.05$. M – molecular weight marker.

cytoplasm of cells treated with recEndoG. Transfection with the EndoG gene did not cause any changes in the ncRNA levels (Fig. 6B). In nuclei and cytoplasm treated with recEndoG, no changes in ncRNA levels were detected (Fig. 6C). From this experiment we can conclude that a constant amount of ncRNA is synthesized and localized in the nuclei and cytoplasm, and that the amount of active EGPO inducing AS Casp-2 is regulated by the activity of EndoG.

DISCUSSION

The first observation of the ability of the EndoG endonuclease to induce AS was shown in our work on the induction of apototic endonucleases upon overexpression of EndoG [29]. A decrease in the expression of deoxyribonuclease I (DNase I) mRNA was found, while the amount of protein remained unchanged. Later, the induction of AS pre-mRNA by DNase I explained this effect. Similarly, induction of the AS pre-mRNA of the catalytic subunit of telomerase hTERT caused the death of tumour cells [16] and lymphocytes [18]. The effect of death and malignant transformation of human or mouse lymphocytes under the influence of EndoG-induced AS *hTERT* has been described [30–36]. The molecular mechanism of action of EndoG on the AS system has been described for primary transcripts of the hTERT gene [22, 37] and DNase I [17]. Sequencing of total RNA from human and mouse lymphocytes after EndoG induction identified a number of other genes whose AS was altered [38].

In this paper we have studied AS, which is the result of the RNase activity of EndoG. The mechanism of AS can be described by the following hypothetical scheme: pre-mRNA is synthesized from the coding strand of the *Casp-2* gene, and ncRNA of 1016 nucleotides in size is synthesized from the template (non-coding) strand (Fig. 7A). EndoG excises from the ncRNA a 60-mer RNA oligonucleotide EGPO, complementary to the Casp-2 pre-mRNA at the junction of exon 9 and intron 9 (Fig. 7B). The interaction of EGPO and Casp-2 pre-mRNA causes AS and

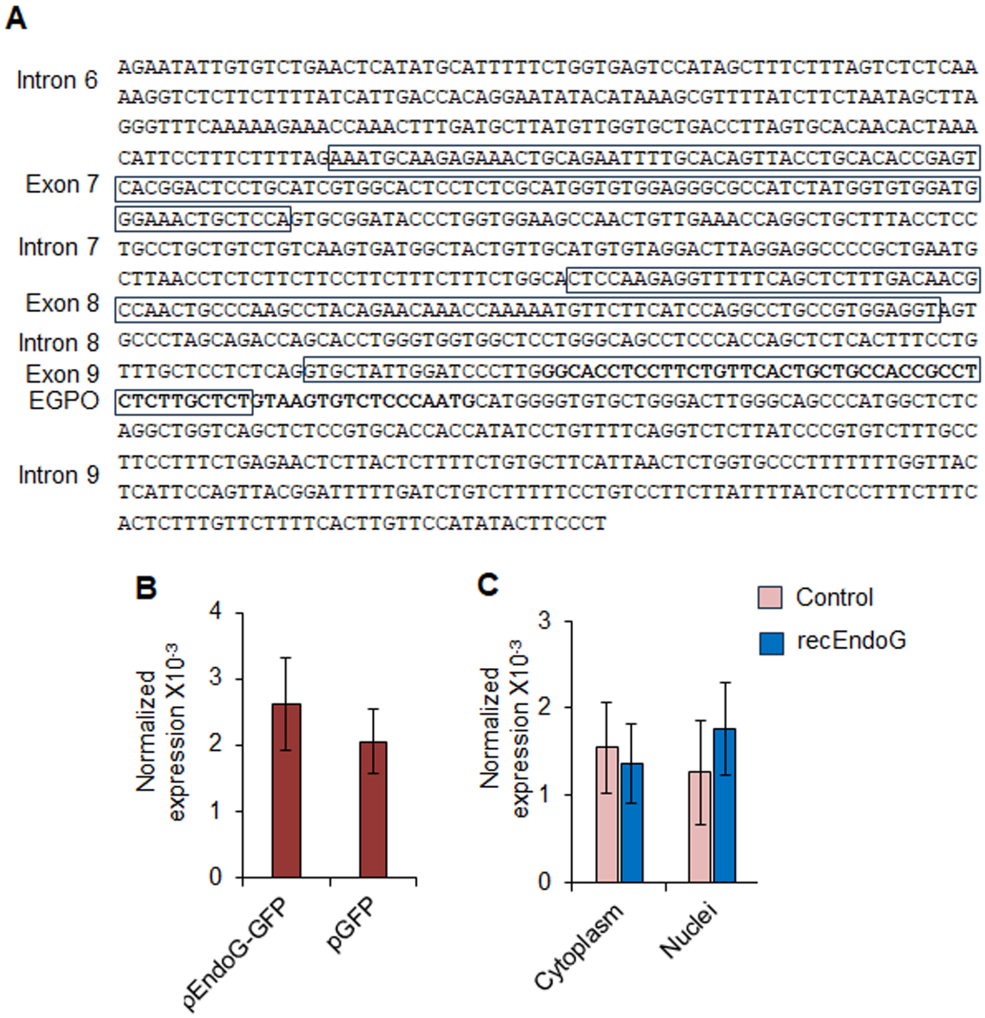

Figure 6. Nucleotide sequence of ncRNA and its synthesis level. (**A**) Nucleotide sequence of ncRNA determined by Sanger sequencing. The regions of the mRNA corresponding to introns and exons and the location of EGPO are shown. The level of ncRNA synthesis in (**B**) transfected $CD4^+$ T cells and in (**C**) the cytoplasm and nuclei of cells after treatment with recEndoG. Exons are boxed. EGPO is shown in bold.

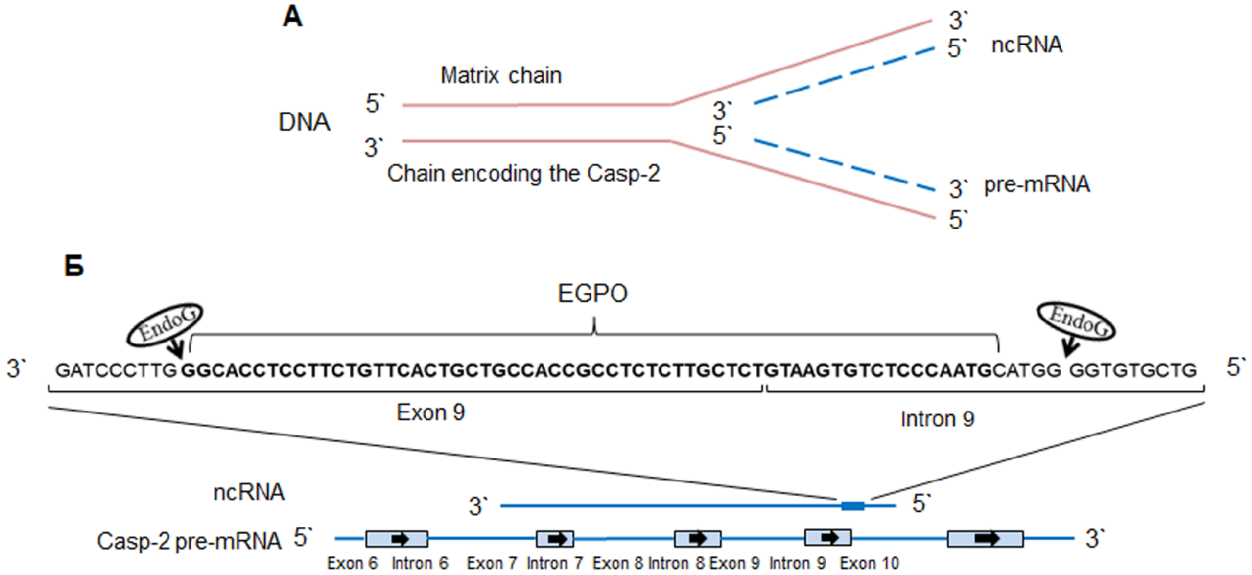

Figure 7. Hypothetical scheme of the described mechanism. (**A**) Scheme of the location of the synthesis sites of ncRNA and *Casp-2* pre-mRNA. (**B**) Hypothetical scheme of the location of ncRNA and *Casp-2* during the induction of AS by the endonuclease EndoG of *Casp-2* pre-mRNA (see explanations in the text).

appearance of Casp-2S. This mechanism is confirmed by the following facts presented in this paper. Increased synthesis of EndoG caused an increase in the expression of Casp-2S and a decrease in the expression of the Casp-2L form of Casp-2, resulting in a decrease in the protease activity of this enzyme [5]. Treatment of the cytoplasm and nuclei of cells with recEndoG caused an increase in the expression of Casp-2S mRNA and a decrease in Casp-2L mRNA. RecEndoG cleavage of total RNA from cell nuclei and subsequent incubation of this RNA with nuclei resulted in the appearance of Casp-2S in these nuclei. Since incubation of nuclei with recEndoG-treated DNA did not result in *Casp-2* AS, these data indicate that the factor causing AS is ncRNA and that its origin is due to the RNase activity of EndoG (since the action of RNases 1 and A did not cause AS). This suggests that the active RNA oligonucleotide (EGPO) is excised from the ncRNA by EndoG.

We believe that the most likely origin of the ncRNA is its synthesis on the template (non-coding) strand of the *Casp-2* gene. The approximate size and nucleotide sequence of EGPO was determined using the method of hybridisation of DNA oligonucleotides with the putative EGPO. This method allowed the size of EGPO to be determined with a minimum error of 12 nucleotides from the 3′ and 5′ ends. Reducing the size of the DNA oligonucleotides decreased their specificity for EGPO and did not lead to inhibition of AS. There are G-rich regions at the boundaries of EGPO (GGG at the 3′ and GGGG at the 5′ end of the molecule), which are likely to be the site of action of EndoG. Artificially synthesised EGPO caused activation of AS *Casp-2* in the nuclei of $CD4^+$ T cells and $CD4^+$ T cells themselves during transfection and led to a decrease in the protease activity of Casp-2.

Previously, the identification of active oligonucleotides produced by EndoG and capable of modulating AS, as well as their specific sites on target pre-mRNAs, allowed their use to modulate cellular processes [19]. Switching splice oligonucleotides have been used to suppress telomerase activity and tumor cell proliferation [39], suppress Casp-2 activity [23] and apoptosis in human T lymphocytes [40], determine the cytoprotective activity of polyamines [41] and modulate the proliferative and suppressor activity of human regulatory T cells [42].

In our work, EGPO expression increased when cells were transfected with the EndoG gene and treated with recEndoG in the cytoplasm and nuclei. To determine the nucleotide substrate for the action of EndoG in the production of EGPO, we used total RNA sequencing, which allowed us to determine the nucleotide sequence of ncRNA. The expression of ncRNAs in cells did not depend on the action of EndoG. This indirectly indicates that the amount of EGPO produced is regulated by EndoG activity, but not by the level of ncRNA expression.

The results of the study of EndoG-induced AS provided the basis for studies of the influence of AS on cellular processes. The role of AS hTERT on the proliferation of human [43–46] and mouse [47] regulatory T cells, as well as its role in the proliferation of $CD4^+$ T lymphocytes [48], has been determined.

CONCLUSIONS

AS Casp-2 is a regulator of the activity of this enzyme and determines the development and inhibition of apoptotic processes in cells; however, the mechanism of regulation of AS has not been fully studied. The result of this work was the description of several phenomena. The ability of the apoptotic endonuclease EndoG to induce AS of *Casp-2* mRNA in the nuclei and cytoplasm of cells was demonstrated. The small RNA EGPO formed from ncRNA has been shown to cause AS by a mechanism similar to that of splicing-switching oligonucleotides. Thus, in this paper we have shown that the mechanism of action of EndoG on the AS system of pre-RNA Casp-2 is similar to that of hTERT [16] and DNase I [17]. Understanding the mechanism of action of EndoG on the AS system has provided direction for work to determine the influence of AS and apoptotic enzymes on proliferative processes in lymphocytes.

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COMPLIANCE WITH ETHICAL STANDARDS

A group of 12 conditionally healthy donors were recruited from the outpatient clinic of the Neurology Research Centre. All patient procedures were performed in accordance with the 1964 Declaration of Helsinki and its subsequent amendments, and the ethical standards of the National Research Committee. The study was approved by the Ethics Committee of the Neuroscience Research Centre (protocol number 12-3 dated 28 December 2020). Written informed consent was obtained from all donors participating in the study.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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ИНДУКЦИЯ АЛЬТЕРНАТИВНОГО СПЛАЙСИНГА КАСПАЗЫ-2 АПОПТОТИЧЕСКОЙ ЭНДОНУКЛЕАЗОЙ EndoG

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Каспаза-2 (Casp-2) — фермент, регулирующий развитие апоптотических процессов в клетке. Пре-мРНК Casp-2 подвергается альтернативному сплайсингу, в результате которого образуются белковый продукт Casp-2L, проявляющий каталитическую активность, и неактивный Casp-2S вариант. Механизм альтернативного сплайсинга пре-мРНК Casp-2 изучен недостаточно полно, однако известно, что апоптотические эндонуклеазы участвуют в регуляции активности Casp-2. Целью данной работы было изучение связи апоптотической эндонуклеазы EndoG и Casp-2 и роли EndoG в регуляции альтернативного сплайсинга пре-мРНК Casp-2. Обнаружена взаимосвязь между экспрессией EndoG и сплайс-форм Casp-2 в $CD4^+$ и $CD8^+$ Т-лимфоцитах человека, которая корреляция усиливалась при инкубации клеток с ингибитором топоизомеразы II этопозидом. Повышение экспрессии Casp-2S сплайс-варианта обнаружено при сверхэкспрессии EndoG в $CD4^+$ Т-клетках, при обработке клеточной цитоплазмы и ядер рекомбинантной EndoG, а также при инкубации ядер с подвергнутой протеолитическому расщеплению рекомбинантной EndoG РНК. Альтернативный сплайсинг Casp-2 индуцировали 60-членным РНК-олигонуклеотидом в изолированных клеточных ядрах и при трансфекции клеток. Идентифицирована длинная некодирующая РНК размером 1016 оснований, из которой образуется активный РНК-олигонуклеотид. На основании полученных результатов предложен следующий механизм сплайсинга мРНК: с кодирующей цепи гена Casp-2 синтезируется пре-мРНК, а с матричной цепи синтезируется длинная не кодирующая РНК. EndoG вырезает из длинной некодирующей РНК 60-членный РНК-олигонуклеотид, комплементарный пре-мРНК Casp-2, в месте соединения экзона 9 и интрона 9, взаимодействие которых вызывает индукцию альтернативного сплайсинга.

Полный текст статьи на русском языке доступен на сайте журнала (http://pbmc.ibmc.msk.ru).

Ключевые слова: EndoG; альтернативный сплайсинг; каспаза-2; Т-лимфоциты

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